Mechanism of Nitrofen Teratogenesis

by Jeanne M. Manson*

Nitrofen (2,4-dichloro-4'-nitrodiphenyl ether) is an herbicide with potent teratogenic activity in rats. When administered at doses as low as 0.15 mg/kg/day during organogenesis, abnormal development of the heart, kidneys, diaphragm, and lung occurs. The specific pattern of visceral malformations produced in the absence of overt maternal toxicity or embryolethality/cytotoxicity suggest that the compound perturbs processes unique or highly selective for embryonic differentiation. Despite findings of metabolic activation to mutagenic intermediates and carcinogenic activity in adult rodents, several lines of evidence indicate that teratogenicity is not based on mutagenic insult to the embryo. Rather, evidence is accumulating that nitrofen exerts a teratogenic effect via alterations in thyroid hormone status. The premature and pharmacologic exposure of the embryo to a nitrofen-derived thyromimetic challenge is believed to be the cause of abnormal morphogenesis of the heart, lungs, kidneys, and diaphragm. The parent compound itself could directly bind to embryonic nuclear receptors for T_3, leading to altered differentiation of target organs. Alternatively, increased availability and placental transport of free thyroid hormones in the maternal compartment could be the source of thyromimetic challenge to the embryo. Overall, these studies indicate that, in the case of nitrofen, the mode of teratogenic activity is uniquely different from the mode of adult toxicity.

Introduction

Previous speakers have presented findings on a variety of agents well-established in laboratory animal models as teratogens. This has permitted relatively direct and extensive examination of mechanism and site of action. In some cases, *in vivo* pharmacokinetic and biotransformation have yielded information critical to understanding teratogenic potency. In other cases, *in vitro* approaches have provided the necessary isolation of embryonic tissues to gain insight into the cellular and molecular mechanism of action. In this paper, I will present findings from studies conducted in my laboratory on the teratogen, nitrofen. When we first began studying this agent approximately six years ago, we had very little information on its adult or developmental toxicity. Consequently, much effort went into first defining the developmental toxicity in rodents, and then into examining a number of parameters potentially related to mechanism of action. This was a multidisciplinary process that included examination of the *in vivo* response to the agent, its distribution and metabolism in pregnant animals, and finally insight into the mechanism and site of action. The main intent of this paper is to describe the approach we took in starting from the beginning to define the developmental toxicity of nitrofen. This will hopefully provide a comprehensive perspective on the multilayered approach required to understand the teratogenic properties of an individual agent.

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Background

Nitrofen (2,4-dichloro-4'-nitrodiphenyl ether) is a selective contact herbicide used on a variety of food crops for pre- and post-emergence control of annual grasses and weeds. It is a member of the chlorophenoxy class of herbicides and is also called nitrophene, TOK, TOK E-25, and Nip (1). Food tolerance levels ranging from 0.02 to 0.75 ppm have been established for a number of commodities including vegetables, milk, rice, poultry and meats (2). Technical grade nitrofen contains approximately 95% active ingredient with impurities consisting of approximately 3% p-chloronitrobenzene, 1% dichlorophenol, and 1% unknown. Woolson et al. (3) specifically determined that technical grade nitrofen lacked 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) contaminants.

Nitrofen is spread on soil surfaces and is adsorbed onto weeds as they emerge. The compound is activated by sunlight and kills weeds by inhibiting photosynthesis. Activation for herbicidal activity appears to involve, but not be exclusively limited to, cleavage at the ether linkage to form dichlorophenol and nitrophenol (4). The biochemical mechanism of herbicidal activity is based upon interference with both oxidative and photosynthetic phosphorylation in mitochondria and chloroplasts of plants. Nitrofen and other diphenyl ether herbicides have been shown to inhibit chloroplast noncylic electron transport by removing or inactivating an electron carrier associated with photosystem II of photosynthesis, or the Hill reaction. Mitochondrial respiration appears to be disrupted at several sites along the electron transport chain (5).

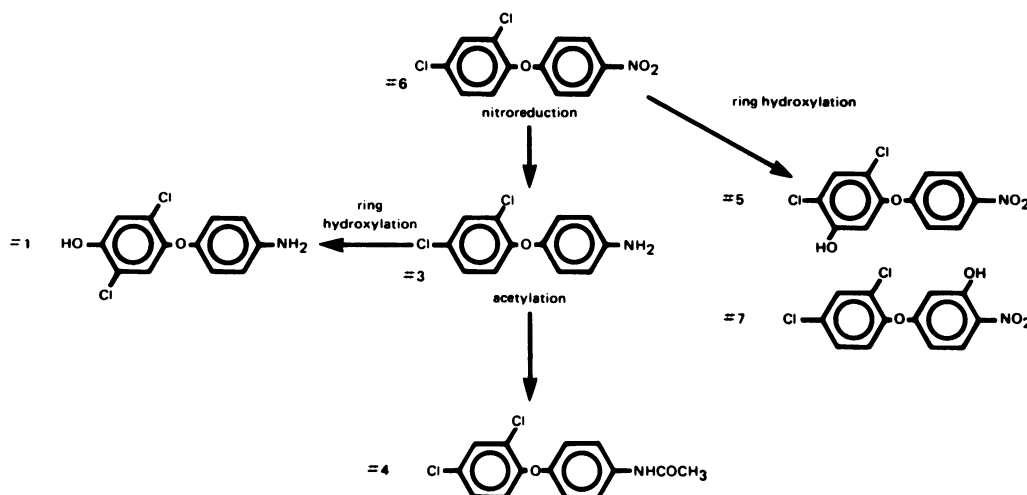## POSTULATED SCHEME OF NITROFEN METABOLISM IN THE RAT

FIGURE 1. Postulated scheme of nitrofen metabolism in the rat with major metabolites depicted. Adapted from Brown and Manson (8).

Approximately 20 different metabolic species, including intermediates and conjugates, have been identified in adult rats after nitrofen exposure. Major metabolites include hydroxylation, nitroreduction and acetylation products, all of which have intact ether linkages (Fig. 1) (6–8). The majority of administered compound is excreted in the urine (23.6%) and feces (73.9%), with approximately 1% taken up by tissues. The postulated route of metabolism in the rat consists of nitroreduction of the parent compound to the 4'- amino derivative (metabolite 3) which is formed in the GI tract and liver with oral exposure. The 4'-amino derivative is rapidly and extensively conjugated to the 4'-acetylamine (metabolite 4) which is retained at high levels in the liver and fat. Alternatively, the 4'-amino derivative and intact parent compound can be hydroxylated, resulting in metabolites 1, 5, and 7.

Nitrofen is not acutely toxic in laboratory animals and has oral LD_{50} values ranging from 2.4 to 3.6 g/kg in rats and mice (1). In subchronic dietary toxicity studies, dose-related increases in liver, testes and kidney weights occurred at 500 ppm and higher. Elevated liver weight and induction of cytochrome P-450 levels have been consistent, early signs of low-dose subchronic exposure. In a cancer bioassay of technical grade nitrofen mixed in the diet with 2% corn oil, pancreatic ductal carcinoma was observed in female rats and hepatocellular carcinoma in mice of both sexes (9). When the bioassay was repeated (10), there was no positive association between treatment and tumor-incidences in rats of either sex. The incidences of hepatocellular adenomas and carcinomas in male and female mice, however, were statistically elevated at all treatment levels. Based on results from these 2 studies, IARC (11) clas-

sified technical grade nitrofen as a carcinogen in experimental animals.

Draper and Casida (12) have examined the mutagenicity of nitrofen in the Salmonella TA 100 strain with metabolic activation. They found that reduction of the $-NO_2$ group on the parent compound is the principal pathway of metabolism with rat liver S9 preparations under anaerobic conditions. The metabolic reduction occurred via formation of highly reactive nitroso (-NO) and hydroxylamine (-NHOH) intermediates to form the 4'-amino ($-NH_2$) metabolite. Under aerobic conditions microsomal oxidation converted the amino (- NH_2) to the nitroso (-NO) intermediate. They concluded that the parent compound is a promutagen, and that the -NO, -NHOH and $-NH_2$ intermediates are mutagens in the Ames assay with TA100. The -NO and -NHOH intermediates were postulated to play a role in the carcinogenicity and teratogenicity of nitrofen.

Developmental Toxicology

The reproductive and developmental toxicity of nitrofen have been examined in over 25 studies published over the last 10 years (1). No adverse effects have been observed on male or female fertility or on developmental process up to implantation. Exposure of pregnant rodents during organogenesis, however, has uniformly resulted in severe developmental toxicity. When administered on days 8–18 of gestation to rats, newborns lacking external malformations develop signs of respiratory distress, become cyanotic and die shortly after birth (13) (Table 1). Treatment on day 11 of gestation (sperm day equal to day 1) caused the greatest reduction in neonatal survival (7). When administered from 75–

Table 1. Effect of nitrofen on neonatal survival when administered on days 8–18 of gestation.[a]

Treatment, mg/kg/ day	No. of litters	Relative maternal wt gain, %	Birth wt, g	Percent survival to 1 hr	Percent survival to 24 hr	Percent survival to 25 days
Corn oil	5	18	6.3 ± 0.2	98	98	98
20	5	18	6.3 ± 0.1	95	93	92
31.2	5	13	5.8 ± 0.1*	43	42	0
50	5	14	5.6 ± 0.1*	41	29	7

[a] Nitrofen was administered orally as a solution in corn oil on days 8–18 of gestation to Sprague-Dawley rats. Adapted from Stone and Manson (13).
*$p < 0.05$.

Table 2. Prenatal toxicity of nitrofen exposure.[a]

Dose, mg/kg	% Increase in maternal wt, day 1–21	% Implants, dead/ resorbed	No. of live fetuses	Fetal body wt, g
0	37	3.7	13.4	5.63
75	39	3.1	12.6	5.46
150	40	3.5	11.0	5.49
250	35	4.1	10.8	5.37
400	31	5.8	10.4	4.70*

[a] Nitrofen was administered by gavage on day 11 of gestation and uterine contents examined on day 21. Adapted from Costlow and Manson (7).
*$p<0.05$ (13).

Table 3. Visceral malformations induced by prenatal nitrofen exposure[a]

Dose, mg/kg	% live fetuses with Diaphragmatic hernias	Hydronephrosis	Heart anomalies	Any visceral malformation
0	0	0	0	0
75	2	22	26	45
150	10	41	38	83
250	20	48	53	96
400	21	62	88	100

[a] Conditions given in Table 2.

400 mg/kg on day 11, no adverse effects on maternal weight gain, postimplantation survival or number of live fetuses at term were obtained, although fetal weight was significantly depressed at the highest dose (Table 2). Skeletal and visceral examination of term fetuses exposed on day 11 revealed dose-related increases in major malformations of the heart, kidneys and diaphragm (Table 3) as well as lung hypoplasia. An unusual aspect of these findings was that the compound induced a specific pattern of visceral malformations alone without causing decreased maternal weight gain, embryolethality or excess cell death in embryonic target organs (14). Skeletal malformations were not observed, even though embryonic limb buds in particular are highly susceptible to teratogenic insult on day 11 of rat gestation. Litter sizes at term were within normal ranges, even at exposure levels that malform the entire litter, indicating that most affected embryos survive to term carrying malformations. These findings indicate that nitrofen exerts a teratogenic effect through specific mechanism(s) of action most likely involving pertubations of

processes unique or highly selective for embryonic differentiation.

Reviews of the literature on the teratogenic properties of nitrofen have revealed some discrepancies in the pattern of major malformations produced with exposure of different rat strains during organogenesis. Kang et al. (15) recently compared the response of different strains of rats [Long-Evans Hooded (LEH), Sprague-Dawley (SD), and "virus antibody negative" Sprague-Dawley (VAN-SD)] to oral exposure of nitrofen at 0, 6.25, 12.50, or 25 mg/kg/day on days 6–15 of gestation. Abnormalities of the lung (hypoplasia), kidneys (hydronephrosis), diaphragm (hernia), and heart (aortic arch anomalies, ventricular septal defects, transpositions of great vessels) were observed in live fetuses at term. The incidence of fetuses with any malformation was nearly identical across dose level for each strain of rat (Fig. 2). There were, however, substantial differences in the pattern of malformations produced within each strain (Fig. 3). The SD and VAN-SD rats responded similarly for all malformations but had significantly higher incidences of diaphragm and lung anomalies than LEH rats. Conversely, LEH rats had significantly elevated levels of kidney anomalies compared to SD and VAN-SD rats. The frequency of heart malformations was generally low and similar across strains at the dose levels employed. These results sug-
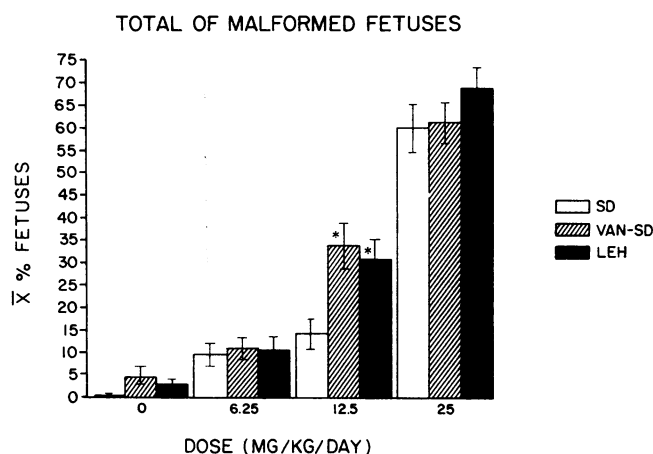
TOTAL OF MALFORMED FETUSES

FIGURE 2. Incidence of fetuses with any malformations in different rat strains. Adapted from Kang et al. (15).
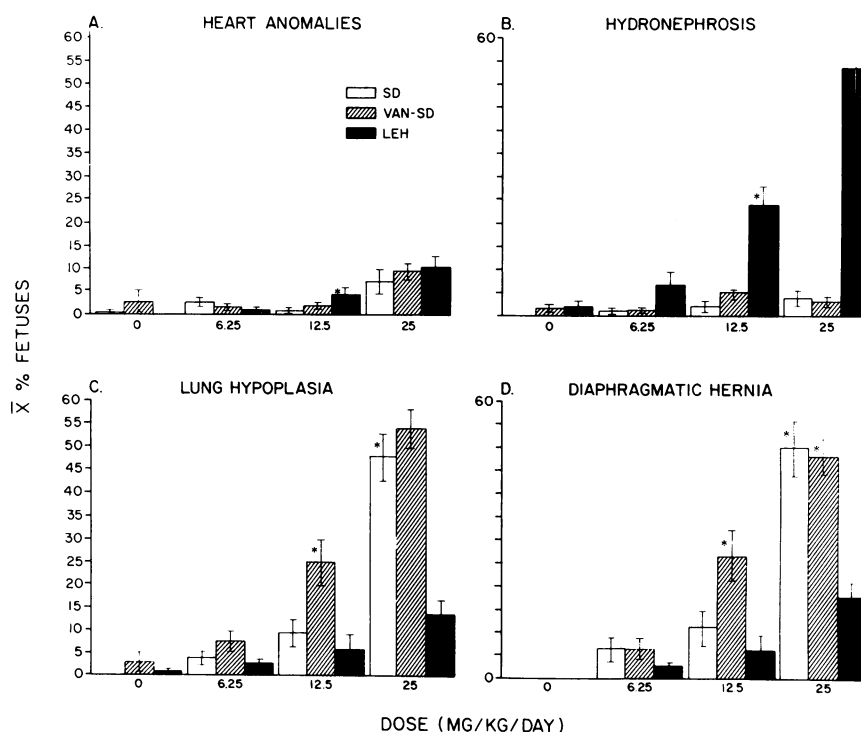
FIGURE 3. Comparison of the malformation pattern produced within each rat strain. Adapted from Kang et al. (15).

gest that while the potency (total percentage of malformed fetuses) of nitrofen is similar in different strains of rats, there are marked strain differences in embryonic target organs affected. This more likely due to genetic differences in embryonic susceptibility than to pharmacokinetics insofar as the potency of nitrofen was similar across rat strains. Results from this study also suggest that lung hypoplasia and diaphragmatic hernias share a common etiology in that they were found together in most fetuses examined from all strains.

A review of the published literature on the developmental toxicity of nitrofen (16) has identified a LOEL (lowest-observable-effect level) of 0.15 mg/kg/day for production of malformations with oral treatment on days 7–15 of gestation in the rat. Comparison of this value to oral LD_{50} values of 2.4 to 3.6 g/kg in rats indicates that the compound is a potent and selective teratogen in rodents. This raises concern about the teratogenicity of other diphenyl ethers, which constitute a major class of pre- and post-emergent herbicides that also includes bifenox, acifluoren, oxyfluorfen, CNP and other candidates. Diphenyl ethers are also being used industrially as components of heat transfer media, solvents, and plasticizers (17).

Distribution and Metabolism of Nitrofen in Pregnant Rats

The distribution and metabolism of nitrofen have been examined in pregnant rats to obtain information on met-

abolic species reaching the embryonic compartment (8,18). Following a single oral administration of radiolabeled nitrofen on day 10 of rat gestation (sperm day = day 0), maternal and embryonic tissues were collected at intervals from 1.5 to 72 hr (Fig. 4). Radioactivity was preferentially accumulated and retained in maternal fat for over 72 hr. Peak levels were reached in other maternal organs at 3–12 hr. The half-life for total radioactivity in maternal plasma was estimated to
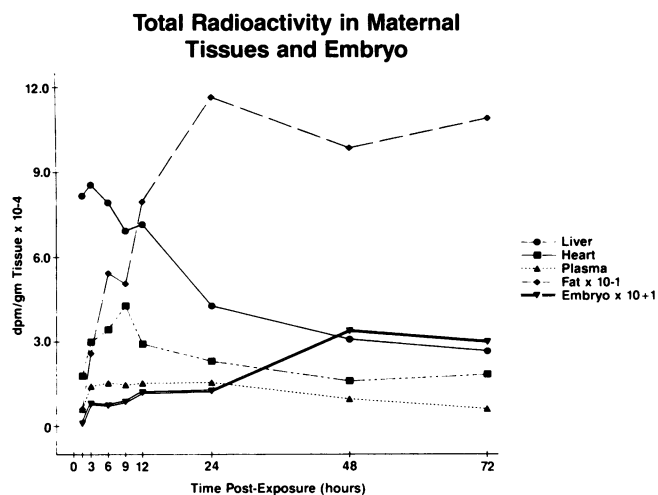

FIGURE 4. Radiolabeled nitrofen was administered on day 10 of rat gestation and maternal and embryonic tissues collected at the indicated time intervals. Adapted from Brown and Manson (8).

be 42 hr. Radioactivity was first detected in the embryonic compartment at 3 hr, and continued to increase to peak levels at 72 hr. Despite the accumulation of radioactivity in the embryo over time, the actual levels found in the embryonic compartment were low, and comprised only 1% of the total 72 hr area under the curve (Table 4).

HPLC analysis of maternal tissues and the embryo/placental complex was carried out to characterize the chemical identity of the radiolabeled material. After initial uptake in maternal tissues (Fig. 5), the parent compound preferentially accumulated in maternal fat starting at 12 hr. After 48 hr, there was a redistribution of parent compound from fat to other maternal organs and the embryonic compartment. The half-life of parent compound in maternal plasma was 68 hr. The 5-hydroxy derivative (see Fig. 1) was the major metabolite found

Table 4. Distribution of ^{14}C-nitrofen in pregnant rats after oral exposures on day 10 of gestation.[a]

Time, hr	Tissue concentration, dpm/mg tissue $\times$ 10^{-4}				
	Liver	Heart	Plasma	Fat	Embryo
1.5	8.2	1.8	0.6	9.3	
3.0	8.6	3.0	1.4	25.9	0.08
6.0	7.9	3.4	1.5	54.4	0.07
9.0	6.9	4.3	1.5	50.6	0.09
12.0	7.2	2.9	1.5	79.6	0.13
24.0	4.3	2.3	1.6	116.8	0.14
48.0	3.1	1.6	1.0	98.7	0.32
72.0	2.7	1.8	0.6	109.3	0.27
72 Hr AUC $\times$ 10^{-5}	31.3	15.7	8.3	67.4	1.5
(Relative % of total)	(25%)	(13%)	(7%)	(54%)	(1%)

[a] The area under the curve (AUC) for each tissue in Figure 5 was calculated to estimate partitioning of total radioactivity in maternal and embryonic tissues. Adapted from Brown and Manson (8).
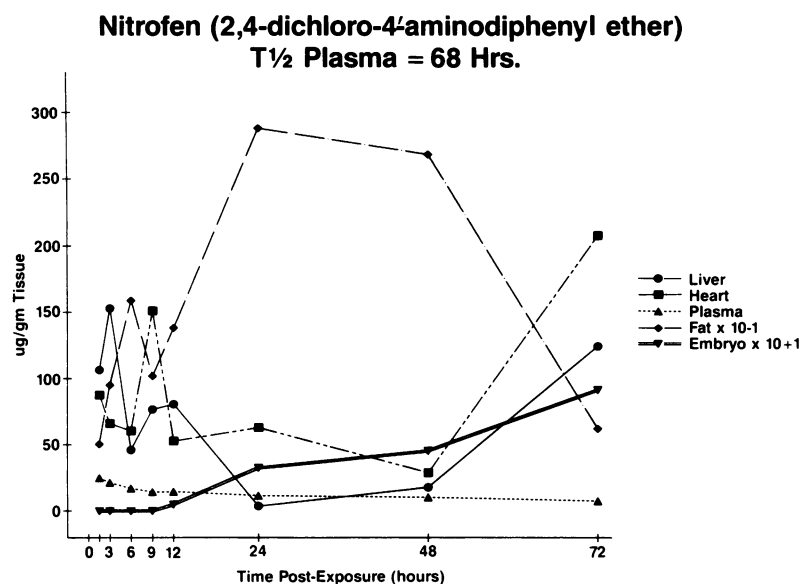
in maternal tissues, while the 4'-amino and 4'-acetylamine derivatives were found at lower levels and all exhibited single phase kinetics (data not shown). The parent compound alone was found in the embryo from 1.5 to 72 hr after maternal exposure (limit of detection 0.03 μg/injection), and levels gradually increased as nitrofen redistributed from maternal fat at 48 hr after exposure.

Despite previous findings of nitroreduction of nitrofen to mutagenic intermediates *in vitro* (*12*), there are several lines of evidence suggesting that the teratogenicity of nitrofen is not based metabolism to mutagenic intermediates which reach the embryo. One is that Costlow and Manson (*18*) administered the 4'-amino metabolite at doses of 65 to 215 mg/kg on day 10 of rat gestation. This metabolite did not influence birth weight, pup weight or neonatal survival to 5 days after birth, parameters which were severely affected by exposure to equivalent doses of the parent compound. HPLC analysis of tissues 6 hr after exposure detected only the 4'-acetylamine, suggesting that the 4'-amino metabolite is preferentially conjugated *in vivo* and not oxidized back through the nitroso pathway.

In addition, Francis (*17*) examined the prenatal toxicity of nitrofen and 5 analogous diphenyl ether compounds with different levels of chlorination. 2,4,5-Trichlorophenyl-4'-nitrodiphenyl ether caused significantly more prenatal toxicity than nitrofen, while 2,4,6-trichlorophenyl-4'-nitrophenyl ether was inactive. Likewise, monochlorinated and unchlorinated derivatives of nitrofen had no adverse effects. These results imply that it is the number and position of chlorine groups on the molecule and not the nitro group that determines teratogenic activity.

In the present study (*8*), the 4'-amino derivative was primarily found in the liver and appeared to be rapidly

Nitrofen (2,4-dichloro-4'-aminodiphenyl ether)
T½ Plasma = 68 Hrs.

FIGURE 5. Distribution of parent compound in maternal and embryonic tissues. Adapted from Brown and Manson (*8*).

conjugated to the 4'-acetylamine which reached relatively high levels in the liver and fat. The absence of both nitroreduction products in the embryonic compartment as well as the prevalence of the 5-hydroxy metabolite in maternal tissue suggest that the metabolic profile *in vivo* may be substantially different than indicated from S9 preparations *in vitro*. Monooxygenation reactions to form the 5-hydroxy derivative of the parent compound appear to be more prevalent with *in vivo* metabolism. The present results do not discount the possibility that mutagenic intermediates derived from nitroreduction are formed in maternal tissues. The absence of the stable endpoint of the nitroreduction pathway, the 4'-amino metabolite, in embryonic tissue suggests that this metabolic pathway may not be relevant to teratogenic induction.

Thyromimetic Mode of Action

Several levels of evidence indicate that nitrofen may exert a teratogenic effect via alterations in thyroid hormone status. One is that nitrofen, as a diphenyl ether compound, has a stereochemical configuration similar to thyroid hormone (Fig. 6). Both possess diphenyl ether ring structures in which the aromatic rings are inclined at an angle of 120° and the planes of the aromatic rings are perpendicular to each other. Manson et al. (*19*) examined the influence of nitrofen exposure on hypothalamic-pituitary-thyroid function in nonpregnant, pregnant, and fetal rats and attempted to relate alterations in thyroid hormone status to teratogenicity. In adult thyroparathyroidectomized (TPTX) female rats, nitrofen exposure at 15 and 30 mg/kg/day for 2 weeks resulted in significant suppression in thyroid stimulating hormone (TSH) levels (Table 5). When a single dose of 250 mg/kg was administered to euthyroid rats, a depression in the release of TSH after a thyrotropin-releasing hormone (TRH) challenge was observed (data not

Table 5. Thyroid hormone levels in female rats after nitrofen exposure for two weeks.[a]

Group	T4, mg/100 mL	T3, mg/mL	TSH, mg/mL
Sham	5.10	0.47	125
TPTX	2.02	0.10	1906
TPTX-15N	2.11	0.15	1173*
TPTX-30N	1.97	0.14	1318*

[a] Female rats were sham operated or thyroparathyroidectomized (TPTX) and treated with 15 or 30 mg/kg/day of nitrofen for 2 weeks. Adapted from Manson et al. (*19*).
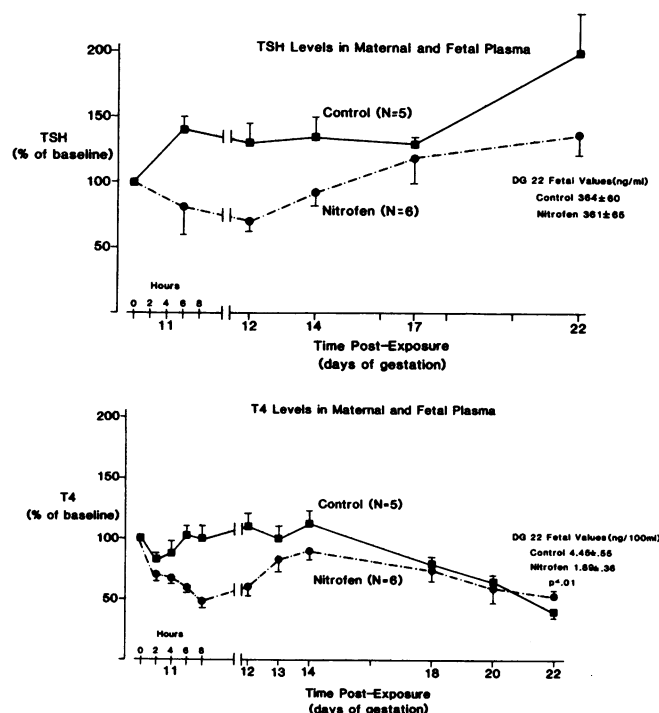* $p < 0.05$.

FIGURE 7. TSH and T_4 levels in maternal and fetal plasma after treatment with nitrofen at 250 mg/kg on day 11 of gestation. Adapted from Costlow and Manson (*18*).
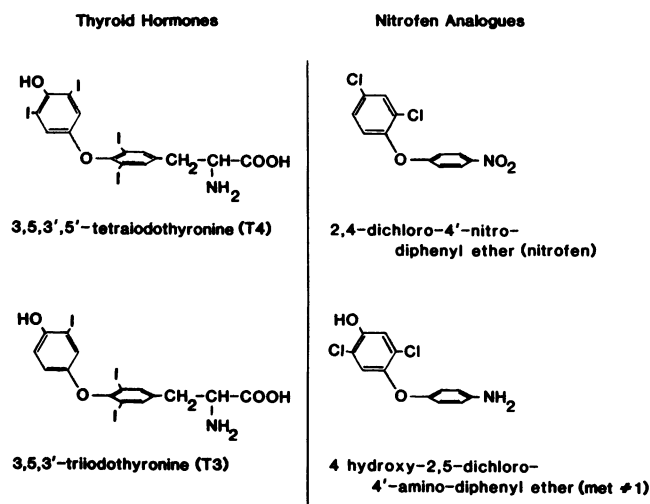
shown). Pregnant euthyroid rats given a single dose of 250 mg/kg on day 11 had significantly depressed TSH and T_4 levels, and fetal T_4 levels were markedly depressed at term (Fig. 7). Coadministration of T_4 with nitrofen to TPTX pregnant rats resulted in a 70% reduction the frequency of malformed fetuses compared with nitrofen exposure alone (Table 6). Heart malformations, in particular, were prevented by coadministration of nitrofen with T_4. These results were interpreted to indicate that nitrofen teratogenicity is mediated, at least in part, by alterations in maternal and/or fetal thyroid hormone status. The premature and pharmacological exposure of the embryo to a nitrofen-derived thyromimetic substance may alter differentiation of the heart, lungs, kidney and diaphragm.

This hypothesis has been pursued in a number of avenues to identify the active agent and the primary site of action. Competitive displacement studies in RIA's for T_4 and T_3 indicated that a nitrofen metabolite (4-

FIGURE 6. Stereochemical structures of thyroid hormones, nitrofen, and metabolite 1. Adapted from Manson et al. (*19*).

Table 6. Protection against nitrofen teratogenicity by thyroxine.[a]

	Sham	TPTX	TPTX-T4	TPTX-N	TPTX-N-T4
No. of litters examined	6	8	5	11	7
No. of fetuses examined	67	76	39	125	73
% Litters with					
Heart anomalies	0	0	0	100	29
Kidney anomalies	0	12	0	73	57
Diaphragm anomalies	0	0	0	27	29
$\bar{X}$, % fetuses with					
Heart anomalies (total)	0	0	0	26.7 ± 2.8	2.4 ± 1.6*
Aortic arch anomalies	0	0	0	12.4 ± 2.7	2.4 ± 1.6
Tetralogy of fallot	0	0	0	4.8 ± 2.9	1.3 ± 1.3
Ventricular septal defect	0	0	0	10.3 ± 3.7	0
Truncus arteriosus communis	0	0	0	1.6 ± 1.6	0
Kidney anomalies	0	3.1 ± 3.1	0	17.0 ± 4.0	8.6 ± 3.3
Diaphragmatic hernias	0	0	0	4.6 ± 2.4	3.1 ± 2.1
Total anomalies, %	0	3.1 ± 3.1	0	39.9 ± 5.3	12.3 ± 3.9*

[a] Rats were sham- or TPTX-operated on day 2 of pregnancy. Thyroid hormone was replaced (TPTX-T4) for the remainder of pregnancy; nitrofen was administered at 25 mg/kg/day on days 9–11 of pregnancy (TPTX-25N); or both nitrofen and T_4 were given together (TPTX-25N-T4) under the same conditions. Adapted from Manson et al. (19).
*$p < 0.05$

hydroxy-2,5-dichloro-4'-amino diphenyl ether, metabolite 1) competed with ^{125}I-T_3 for antibody binding, while the parent compound and remainder of metabolites failed to compete with ^{125}I-T_4 or ^{125}I-T_3 for antibody binding. The degree of crossreactivity of metabolite 1 was relatively low (0.88% relative to T_3) and the antibodies were directed against the 4-hydroxy region of the molecule. Insofar as metabolite 1 is found at very low levels in maternal tissues and has not been detected in the embryonic compartment, the significance of this finding is questionable (19).

A series of in vitro studies have recently been carried out in which the competition of nitrofen and metabolites with T_4 for binding to rat thyroid binding globulin (TBG) was examined (20). Rat TBG was isolated from serum by affinity chromatography followed by size exclusion chromatography. A single protein capable of specific T_4 binding with a molecular weight of 52,700 was isolated. These characteristics are similar to those previously reported for transthyretin, or prealbumin, the major thyroid hormone transport protein in the rat (21). Nitrofen and several metabolites were found to significantly compete with ^{125}I-T_4 for binding to rat TBG (Table 7). Chlorination at the 2,4 position is likely to play some role in this competition insofar as 2,4-dichlorodi-

Table 7. Competition of nitrofen and metabolites with T_4 for rat TBG binding.[a]

Compound	IC$_{50}$, mM	% relative competition ^{125}I-T_4
T_4	0.009	100
Metabolite 7	0.021	42
Metabolite 4	0.031	29
Nitrofen	0.037	27
Metabolite 5	0.155	6
Metabolite 3	—	0

[a] Rat thyroid binding globulin (TBG), or prealbumin, was isolated from serum and competition between nitrofen and metabolites with ^{125}I-T_4 for binding was determined. Adapted from Brown et al. (20).

phenyldichloroethane (o,p'-DDD) and 2,4-dinitrophenol are known to have the same effect (22). The fact that metabolite 3 (2,4-dichloro-4'-aminodiphenyl ether) did not compete, however, indicates that other substituents or stereochemical configurations also play a role.

The competition of nitrofen and several metabolites with T_4 for rat TBG binding does explain at least in part some of the perturbations in thyroid hormone status seen in the maternal compartment. Consequences of this competition are that increased levels of free T_4 would be available for conversion to free T_3. Elevated free T_4 and T_3 would then negatively feed back to the pituitary and decrease maternal TSH release, as has been identified (Fig. 7). Also, the level of total T_4 would decrease insofar as over 98% of circulating T_4 is protein bound, a finding also previously identified in pregnant rats treated with nitrofen (Fig. 7). Overall, there would be higher levels of free thyroid hormone available for exerting a physiological effect in the maternal compartment and also potentially available for transport across the placenta to the embryonic compartment.

The thyromimetic activity of nitrofen was further evaluated by examining biochemical indicators of thyroid hormone exposure in maternal and fetal tissues after nitrofen treatment at 0, 12.5, 25, and 50 mg/kg/day on days 6–15 of gestation in the rat (23). Serum levels of T_4 and T_3 were measured on days 15 through 21 in the dam and on days 19 and 21 in the fetus. Malic enzyme activity and total phospholipid content (surfactant) were also measured in fetal lung as indicators of a thyromimetic challenge to the fetus.

Maternal T_4 levels were initially depressed, as previously reported, but then increased to exceed control values in an apparent negative feedback at term (data not shown). Maternal serum T_3 levels were significantly elevated in all treatment groups on days 17–21 of gestation (Fig. 8). Fetal serum T_4 levels were significantly decreased in a dose-dependent manner on days 19 and 21 (Fig. 9, day 21 alone). Serum T_3 was barely detect-
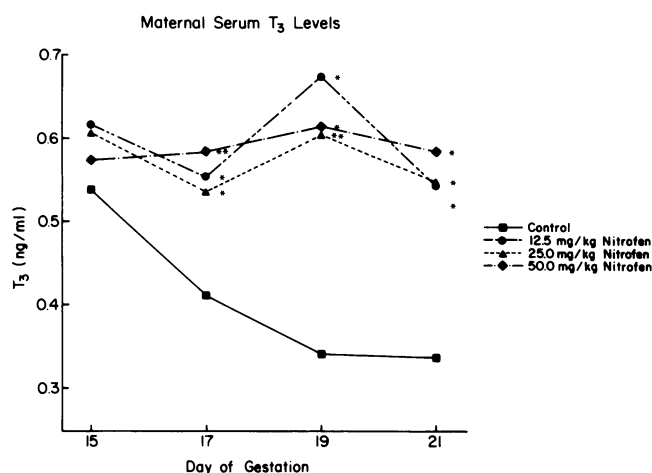
Maternal Serum T$_3$ Levels

FIGURE 8. Maternal serum T$_3$ levels after nitrofen treatment on days 6–15 of pregnancy. Adapted from Tellone and Manson (*23*).

Fetal Serum T$_4$ Levels – Day 21

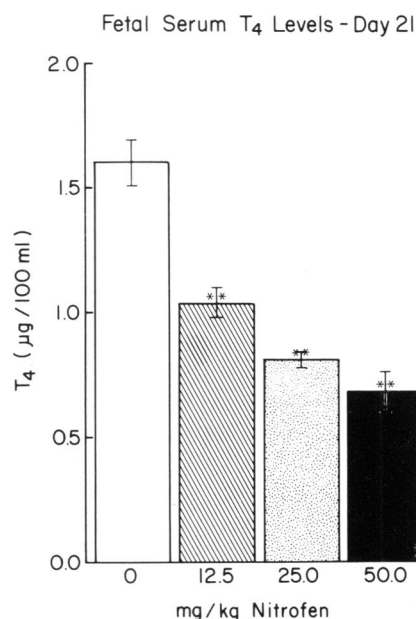FIGURE 9. Fetal serum T$_4$ levels after nitrofen treatment on days 6–15 of pregnancy. Adapted from Tellone and Manson (*23*).

Fetal Serum T$_3$ Levels

FIGURE 10. Fetal serum T$_3$ levels after nitrofen treatment on days 6–15 of pregnancy. Adapted from Tellone and Manson (*23*).

Table 8. Relative fetal lung weight.[a]

Nitrofen exposure, mg/ kg	Relative fetal lung weight			
	Day 15	Day 17	Day 19	Day 21
Control	1.28	2.68	3.49	2.81
	(0.08)	(0.11)	(0.04)	(0.13)
12.5	1.10	2.54*	3.19*	2.28†
	(0.09)	(0.07)	(0.32)	(0.14)
25.0	1.15	2.46*	3.10*	2.21†
	(0.09)	(0.11)	(0.14)	(0.08)
50.0	1.10	2.28†	2.96†	2.09†
	(0.04)	(0.21)	(0.11)	(0.07)

[a] Relative fetal lung weight after nitrofen exposure on days 6–15 of gestation. Adapted from Tellone and Manson (*23*).
* $p < 0.05$.
† $p < 0.01$.

able in control fetuses on day 19; pooled fetal samples from three of five control litters had measurable levels with a mean of 0.05 ng/mL, a level below the valid limit of detection of the assay (0.1 ng/mL) (Fig. 10). Serum from fetuses exposed to nitrofen had barely perceptible increases in T$_3$ levels up to 0.13 ng/mL in the high-dose group on day 19. By day 21, control values for fetal serum T$_3$ were at the limit of detection and slight elevations were observed in fetuses from the two highest dose groups. HPLC analysis indicated that parent compound and traces of metabolite 7 alone (2,4-dichloro-3'-hydroxy-4'-nitrodiphenyl ether) were present in maternal and fetal sera at these time points. Consequentl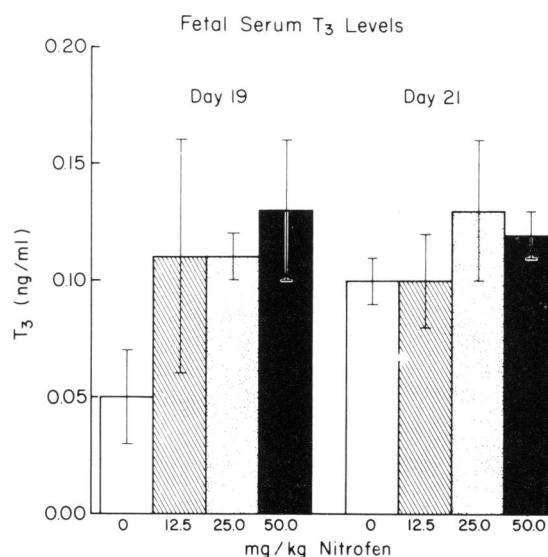y, the elevations in maternal and fetal serum T$_3$ levels were not due to the presence of metabolite 1, which crossreacts with antibodies for T$_3$ and were presumably due to the presence of endogenous T$_3$.

Relative lung weights from nitrofen-treated fetuses in this experiment were significantly depressed from day 17 to day 21 of gestation (Table 8). Total lung phospholipids were measured as an estimate of fetal lung surfactant content. On day 15, phospholipid levels in pooled lungs from control litters were not measurable (Table 9). In treated lungs there was an increase in both the frequency of positive litters and in the level of phospholipid. Slight increases were seen on days 17 and 19 of gestation in treated lungs, and by day 21 no differences in phospholipid levels were evident although treated lungs were markedly smaller than the controls. A similar trend was observed with malic enzyme activity in fetal lungs (Table 10). A dose-related increase in malic enzyme activity was found in treated lungs on day 19 of gestation which was no longer apparent by day 21.

The interpretation of these results is that nitrofen

Table 9. Fetal lung phospholipids—day 15.[a]

| | Phospholipids, μg P/mg protein | | | |
Litter no.	Control	12.5 mg/kg	25.0 mg/kg	50.0 mg/kg
1	0.00	0.00	5.32	7.03
2	0.00	0.00	0.00	4.87
3	0.00	7.20	6.82	7.39
4	0.00	0.00	0.00	0.00
5	0.00	2.41	0.00	0.00
Mean (SEM)	0.00	1.92	2.43	3.86
	(0.00)	(1.40)	(1.51)	(1.63)
Mean (SEM) (Excluding Zeros)	0.00 (0.00)	4.81 (2.40)	6.07* (0.75)	6.43* (0.79)

[a] Total phospholipid levels in fetal lungs after nitrofen treatment on days 6–15 of pregnancy. Adapted from Tellone and Manson (23).
* $p < 0.01$.

Table 10. Malic enzyme activity in fetal lung.[a]

| | Malic enzyme activity, μmole/min/mg protein | |
	Day 19	Day 21
Control		
Mean ± SEM	5.01 ± 0.46	8.41 ± 0.95
12.5 mg/kg		
Mean ± SEM	6.18 ± 0.71	8.72 ± 1.12
25.0 mg/kg		
Mean ± SEM	6.21 ± 0.15	8.21 ± 0.84
50.0 mg/kg		
Mean ± SEM	6.99 ± 0.91*	7.76 ± 1.72

[a] Malic enzyme activity in fetal lungs after nitrofen treatment on days 6–15 of pregnancy. Adapted from Tellone and Manson (23).
* $p < 0.05$.

exposure resulted in a thyromimetic challenge to the conceptus. In the case of the fetal lung, this was manifested as a transient stimulation of lung differentiation at the expense of lung growth. This concept is identical to that developed for explanation of thyroid hormone action on differentiation of the central nervous system. Thyroid hormone alters the course of development in neural tissues by inhibiting proliferation and stimulating differentiation (24). This concept could also be extrapolated to provide an explanation for the heart malformations produced by nitrofen exposure. The resultant thyromimetic challenge could cause a precocious appearance or increase in density of β-adrenergic receptors in the embryonic heart, as has been demonstrated in neonatal and adult tissue (25). Heart malformations, particularly of the arterial outflow tract, have been produced in embryonic chicks via β-adrenergic stimulation, presumably through altered cardiovascular hemodynamics (26). No link can yet be made between a thyromimetic challenge and kidney and diaphragm malformations. In general, little is known about the mechanisms whereby teratogens alter the differentiation of these tissues.

Current efforts are directed toward understanding what constitutes the thyromimetic challenge to the fetus with nitrofen exposure. It has been conventionally held that thyroid hormone is not important in rodent development until the late fetal and postnatal period. This is based on findings that the fetal thyroid gland does not

begin to synthesize and release thyroid hormone until day 18 of gestation, and that the placenta is impermeable to transfer of thyroid hormone. Consequently, development is believed to occur in a thyroid hormone-free environment until the fetal thyroid is functional (27). These findings have recently been challenged by findings that both T_4 and T_3 cross the placenta in significant amounts during organogenesis in the rat. Higher levels of these hormones were found in embryo trophoblasts on days 10–12 of gestation than in the conceptus from days 13–18. After day 18, levels were markedly increased from fetal thyroid gland production (28). Likewise, maternal thyroidectomy or iodine deficiency caused a decrease in thyroid hormone levels in the conceptus both before and after onset of fetal thyroid secretion (29). Perez-Castillo et al. (30) have recently shown that nuclear receptors for T_3 were present as early as day 13 of gestation in whole rat embryo homogenates. The receptor could be measured in individual organs from day 14 (brain) or day 16 (heart, liver, and lung) onward. The presence of both T_4 and T_3, presumably from maternal origin, as well as the nuclear receptor for T_3 suggest that thyroid hormones could influence development from day 13 onward.

With this recent evidence on the presence of thyroid hormone and its nuclear receptor during late organogenesis, there are at least two possible explanations for the thyromimetic challenge that occurs with nitrofen exposure to produce abnormal development. One is that the parent compound itself, or less likely a metabolites, directly interacts with nuclear receptors for T_3 in the embryo. Precocious differentiation of those tissues capable of responding to thyroid hormone (lung, heart) would occur and lead to abnormal morphogenesis.

Another explanation is that primary effect of nitrofen is to cause alterations in maternal thyroid hormone status, possibly due to competition with T_4 for rat TBG binding. Elevated levels of free thyroid hormones would be available to cross the placenta, and maternally derived hormones would constitute the thyromimetic challenge to the conceptus. This hypothesis is supported by findings that the same pattern of depressed T_4 and elevated T_3 in the maternal compartment is also found in the fetal compartment after nitrofen exposure on day 6–15 of gestation. These two possibilities are now being explored by examining competition of nitrofen and metabolites with T_3 for nuclear receptor occupancy as well as by monitoring levels of thyroid hormone in the embryonic compartment after *in vivo* exposure to nitrofen.

Conclusions

Nitrofen is a potent teratogen in rats that produces abnormal development of the heart, kidneys, diaphragm, and lung when administered during organogenesis. The specific pattern of visceral malformations that occur in the absence of overt maternal toxicity or embryolethality/cytotoxicity suggest that the compound perturbs processes unique or highly selective for embryonic differentiation. Despite findings of metabolic

activation of nitrofen to mutagenic intermediates, several lines of evidence indicate that teratogenicity is not based on mutagenic insult to the embryo. Rather, evidence is accumulating that nitrofen exerts a teratogenic effect via alterations in thyroid hormone status. The premature and pharmacological exposure of the embryo to a nitrofen-derived thyromimetic substance is believed to be the cause of abnormal morphogenesis of the heart, lungs, kidneys, and diaphragm. The proximate teratogen could be the parent compound itself which directly binds to embryonic nuclear receptors for T_3, leading to altered differentiation of target organs. Alternatively, increased availability and placental transport of maternal thyroid hormones could be the proximate source of the thyromimetic challenge to the embryo.

An important perspective to be gained from these studies is that the developmental toxicity of an agent can be considerably different and more complex than its adult toxicity. In the case of nitrofen, its carcinogenic activity can be explained by metabolism to mutagenic intermediates. Its developmental toxicity, however, is most likely based on an entirely different mechanism of action involving a thyromimetic challenge to the conceptus. Factors contributing to the specialty of developmental toxicity are the complex interactions that occur between maternal, placental and embryonic systems and the unique susceptibility of embryonic tissues. Consequently, assumptions cannot be made that the mode of developmental toxicity is identical to the mode of adult toxicity, even for agents whose adult toxicity has been well defined.

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